The *Leishmania* membrane bound Histidine Acid Phosphatases (HAcPs) as putative virulence factors; Characterization of the *Ld*MAcP, a Histidine Acid ecto-phosphatase from *L. donovani*

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Introduction

Leishmania* spp.** are protozoan parasites of the Trypanosomatid order. The life cycle of the parasite includes a non-intracellular stage (promastigote phase) in the insect host and an obligatory intracellular stage (amastigote phase) inside the phagosomes of mammalian host phagocytes where they proliferate and establish a replicative niche (**Fig. 1**). ***Leishmania donovani is the causative agent of the potentially fatal disease, visceral leishmaniasis (VS-Kala azar), in humans.

The superfamily of **histidine acid phosphatases** (**HAcPs**) is a large and functionally diverse group of enzymes, split in two branches, which share a conserved signature motif (R**H**GXRXP) in their catalytic core (*Rigden D. J. 2008)*. Human representatives of both branches are of considerable medical interest. Other members of the HAcPs exist in various pathogenic microorganisms (*Mohapatra N.P., 2013*) and their inhibition might have therapeutic value (*Rigden D. J. 2008)*.

Leishmania donovani ***Ld*MAcP** phosphatase belongs to the HAcP superfamily, is membrane anchored and shares high sequence identity with the secreted *Leishmania donovani* acid phosphatases (*Ld*SAcPs) *(Shakarian A. Dwyer DM, et al., 2002)* (**Fig. 2**). *Ld*MAcP is postulated to confer to the acid ecto-phosphatase activity (*Gottlieb M., Dwyer DM., 1981*) (**Fig. 3**) of intact parasites which has been implicated in promastigote virulence (*Sigla N., 1992*).

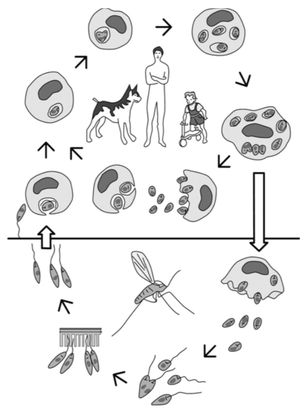


Fig. 1: Life cycle of *Leishmania* spp

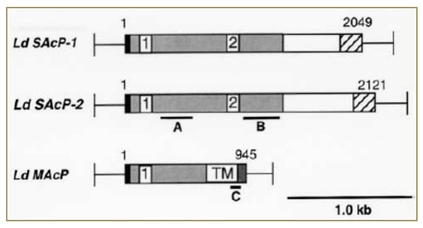


Fig. 2: Diagrammatic representations of the *L. donovani secreted and membrane bound* HAcP coding genes identified by *Shakarian A. Dwyer DM, et al., 2003*.

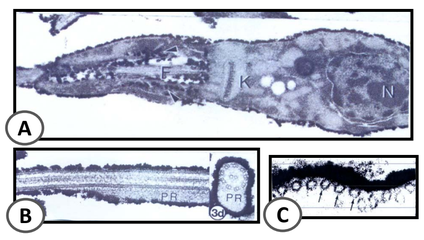


Fig. 3: Intact *L. donovani* promastigotes were incubated with b-glycerophospate that gives an electron dense lead phosphate enzyme reaction product uniformly distributed in the entire surface membrane of *L. donovani* promastigote body (A) and flagellum (B) and on the outer surface of isolated surface membranes (C). Arrows in (C) show the subpelicular MTs. (*Gotlieb & Dwyer 1981*).

Aim of this work was to identify HAcP ecto-phosphatase candidates and elucidate the localization and structural/ functional properties of the ***Ld*MAcP**, as the first candidate for this activity, in order to contribute to the understanding of its role in *Leishmania donovani* life cycle and virulence.

Bioinformatics Results

Membrane bound HAcPs from *Leishmania* spp

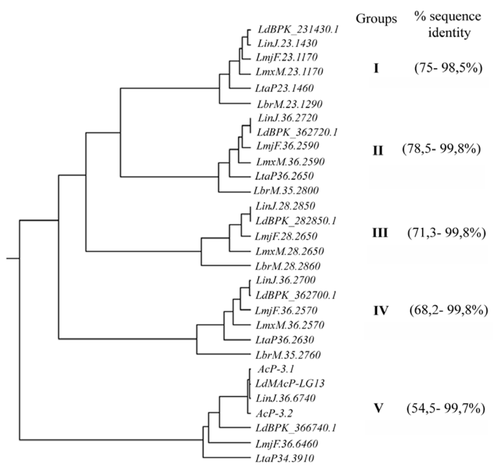


Fig. 4: Rooted phylogenetic tree (UPGMA) with branch length from a multiple sequence alignment of the putative membrane bound HAcPs from *Leishmania* spp. Five groups of *Leishmania* ORFs with the HAcP signature motif, predicted to be membrane bound were identified in the genomes of six *Leishmania* spp. The amino acid sequences were obtained from the GenBank and TriTryp sequence databases.

*Ld*MAcP & *Ld*SAcPs belong to the Branch 2 HAcP subfamily

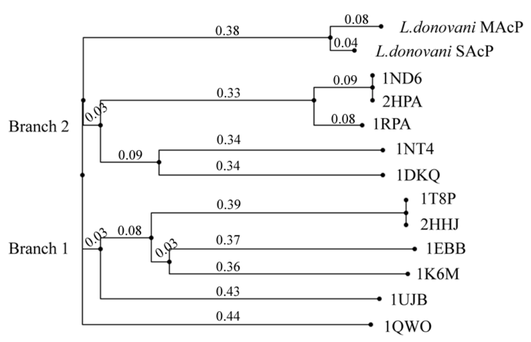


Fig. 6: Consensus phylogenetic tree derived from sequence alignment of *L. donovani* MAcP and SAcP-1 with several members of the HAcP superfamily with known tertiary structures. Proteins shown are the Human Prostatic Acid Phosphatase (1ND6), Human Prostatic Acid Phosphatase Precursor (2HPA), Rat Prostatic Acid Phosphatase (1RPA), *E. coli* Glucose 1-Phosphatase (1NT4), *E.coli* Phytase Acid Phosphatase (1DKQ), Human Erythrocyte 2,3 biphosphoglyceratemutase (1T8P), Human biphosphoglyceratemutase (2HHJ), Phosphoglyceratemutase homolog from *Bacillus stearothermophilus* (1EBB), Human Liver 6-Phosphofructo-2-Kinase/Fructose-2,6–biphosphatase (1K6M), *E. coli* Histidine Phosphatase SixA (1UJB), *A. fumigates* Phytase (1QWO). Branch numbers are indicated. Branch 2 HAcPs (*Rigden D. J. 2008)* appear to enter the secretory pathway Some enzymes remain in the ER, others are found at the cell surface, periplasm or cell wall, and others are simply secreted.

Identification of putative HAc ecto-phosphatases

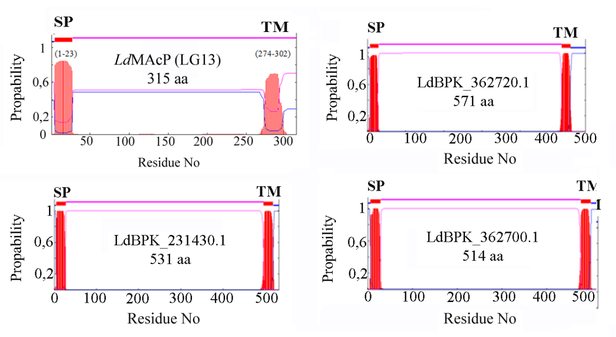


Fig. 5: TMHMM transmembrane prediction profiles of four *L. donovani* membrane bound acid phosphatases. The *Ld*MAcP-protein protein sequence from the *L. donovani* LG13 strain (MHOM/ET/0000/HUSSEN) and three predicted membrane bound HAcPs from the *L. donovani* LDBPK282A1 Nepalese strain were analysed.
SP=secretion signal peptide; TM=transmembrane domain. These protein sequences are predicted to have Type I membrane protein topology and are thus putative ecto-phosphatatases.

The *Ld*MAcP ecto-phosphatase is specific for the *L. donovani* complex

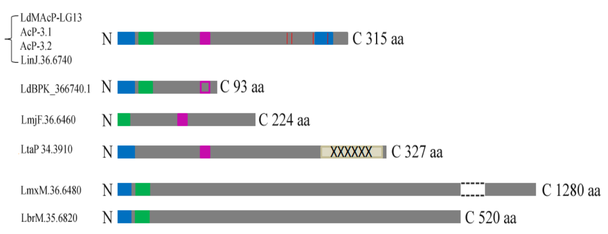


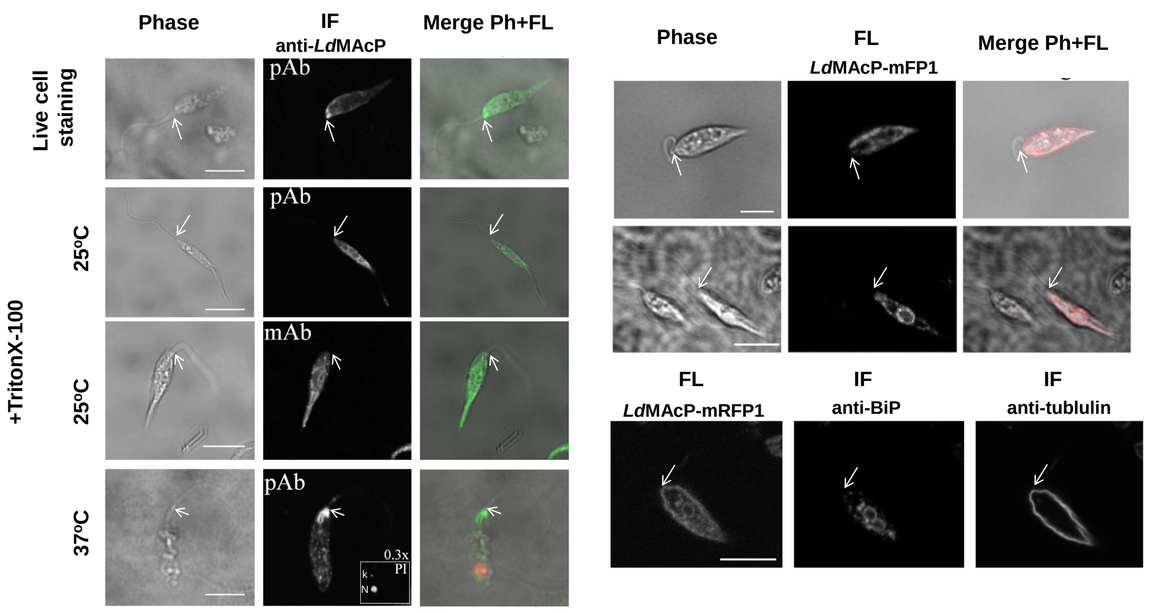
Fig. 7: Schematic representation of ClustalW multiple sequence alignment of *Ld*MAcP orthologs in *Leishmania* spp. All the amino acid (aa) sequences were obtained from the EMBL and TriTrypDB databases except for the aa sequence of the *L. donovani*-LG13 *Ld*MAcP that was generated in this work. The **blue** vertical boxes indicate the putative 23-aa signal peptide (Met1–Ala23) and the putative TM domain (Leu274-Tyr302) of the *L. donovani*-MAcP as predicted by the TMHMM algorithm (Fig 6).
The **green** vertical box indicates the HAcP signature motif.
The **purple** horizontal bar (E73—L84) indicates the peptide sequence used to generate an anti-*Ld*MAcP mAb.
X stands for non identified aa.

Conclusions

- The genomes from six *Leishmania* spp carry coding sequences for four putative Histidine Acid ecto-phosphatases
- The *Ld*MAcP ecto-phosphatase is specific for the *L. donovani* complex and presents strain polymorphisms
- The *Ld*MAcP and r*Ld*MAcP-mRFP1 proteins have an ER-like and surface membrane localization in *L. donovani* parasites
- Overexpression of the *Ld*MAcP in *L. donovani* promastigotes results in an increased acid ecto-phosphatase activity
- The r*Ld*MAcP-His transiently expressed in mammalian cells is targeted to the plasma membrane and has an extracellular topology with ecto-phosphatase activity
- The *L. donovani*-r*Ld*MAcP-mRFP1 promastigotes infect J774 murine macrophages more efficiently than *L. donovani*-pLexsy-sat promastigotes suggesting a possible role of *Ld*MAcP in *L. donovani* infectivity.

Microscopy and Biochemical Results

***Ld*MAcP is localized on the surface of wt *L. donovani* and transgenic *L. donovani*-r*Ld*MAcP-mRFP1 parasites**



iii. Acid ecto-phosphatase activity in live *L. donovani* cells

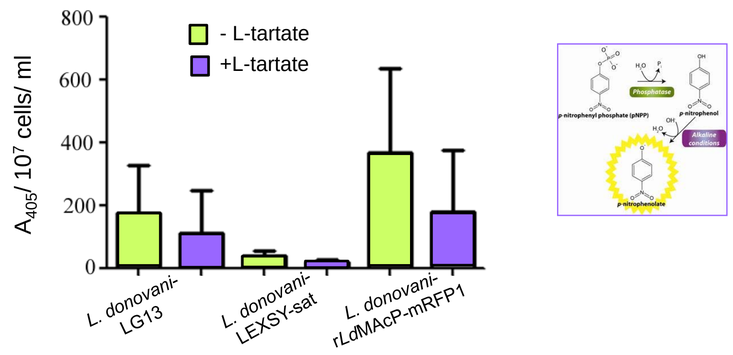


Fig. 8: i. The endogenous *Ld*MAcP is visualized by immunofluorescence (**IF**) in live or fixed -/+ TX-100 treated *L. donovani*-LG13 wt parasites using mouse anti-*Ld*MAcP monoclonal and polyclonal Abs. Nuclei (N) and kinetoplast (k) DNA was stained with propidium iodide. **ii.** The r*Ld*MAcP-mRFP1 fluorescence (**FL**) in *L. donovani*-r*Ld*MAcP-mRFP1 promastigotes at stationary phase of growth was visualized by confocal microscopy. IF co-staining of fixed 0.1% TX-100 treated cells with the anti-BiP (kind gift of Dr J. D. Bangs, U. at Buffalo (SUNY) & 2nd anti-rabbit Alexa633), and the anti-Tubulin (#T5168-Sigma, 2nd anti-mouse Alexa488) Abs shows typical ER and peripheral localization of the r*Ld*MAcP-mRFP1 protein. Acquisition of the red mRFP1 Fluorescence signal was performed separately from the other two fluorescence signals. Arrows point to the begining of the flagelum. Scale bar 4μm. **iii.** Ecto-phosphatase activity was measured in live stationary phase *L. donovani* promastigotes using the p-nitrophenyl phosphate (*p*NPP) (10 mM) as substrate in a 90 mM citrate buffer (pH 4.8). The transgenic *L. donovani*-r*Ld*MAcP-mRFP1 parasites have **~2X & 5X higher** acid ecto-phosphatase activity as compared to the wt and the mock transfected promastigotes, respectively.

Heterologous expression of the r*Ld*MAcP-His in mammalian HeLa cells leads to increase of surface acid ecto-phosphatase activity

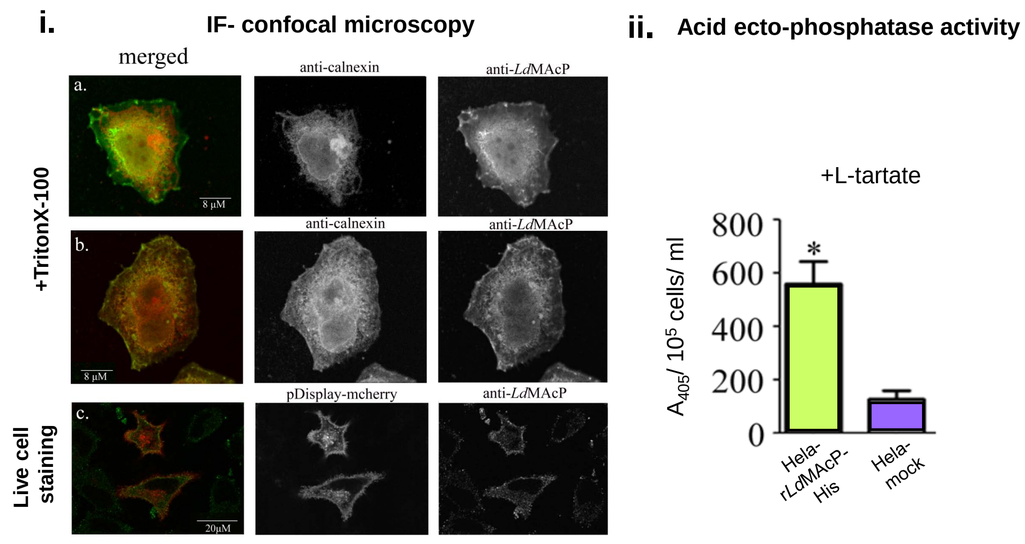


Fig. 9: i. IF analysis of HeLa cells transiently trasnfected with the pTrieX1.1-*Ld*MAcP plasmid. The r*Ld*MAcP-His protein was localized with an anti-*Ld*MAcP (a ,b ,c) mAb in the ER [colocalization with Calnexin (a,b)] and plasma membrane [colocalization with the plasma membrane expressed mcherry, by contrasfection with the pDisplay plasmid (c)]. The extracellular surface topology of the r*Ld*MAcP-His globular domain was confirmed by staining the r*Ld*MAcP-His expressing HeLa cells live (c) with an anti-*Ld*MAcP mouse pAb (c). **ii.** Acid ecto-phosphatase activity of live HeLa cells (measured phosphatas in Fig. 8) transfected with the pTrieX1.1-*Ld*MAcP plasmid was **~6X higher** in the presence of L-tartate acid (inhibitor of acid phosphatases) when compared to HeLa cells mock transfected with the pTriex1.1. plasmid. [*Normalization was performed according to the % of cells expressing the rLdMAcP-His estimated by IF with the anti-His Ab*] .

L. donovani -r*Ld*MAcP-mRFP1 promastigotes show increased infectivity and survival in an *in vitro* infection assay *of* J774 mouse macrophages

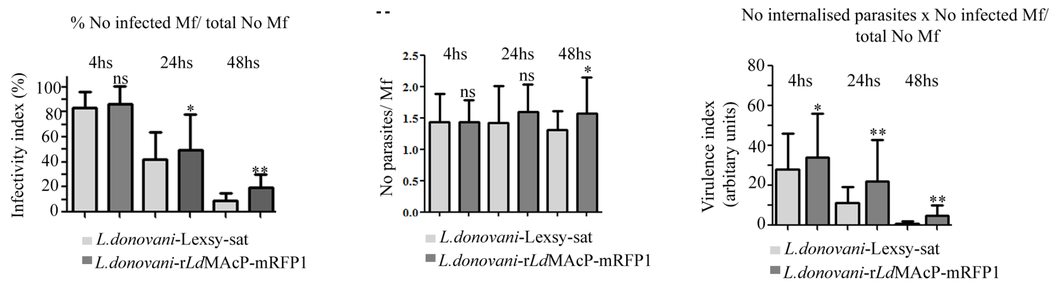


Fig. 10: The *L. donovani*-pLexsy-sat-r*Ld*MAcP-mRFP1 promastigotes show higher efficiency to infect and survive in J774 mouse macrophages (Mf) in culture than parasites carrying the empty vector, up to 48hs post infection. The infectivity was evaluated by incubating stationary phase promastigotes with macrophages for one hour, removal of the free parasites by excessive washing and further incubation for 3hs, 23hs and 47hs. Enumeration of the infected cells was performed by IF staining of non-internalized parasites with a mouse anti-LG13 serum and the anti-mouse Alexa Fluor® 546, in the absence of TX-100 and subsequent fixation & staining of both internalized and non-internalized parasites with the same Ab in the presence of 0,1% TX-100 and as 2nd the anti-mouse Alexa Fluor® 488 *(i.e Internalized parasites are visualised only with green FL while external with both red + green FL*). Parasites and macrophages were enumerated by the use of the Icy digital image analysis software.
i. infectivity index **ii.** Mean no of parasites/Mf **iii.** Virulence index.

Funding: This work was funded by an **Hellenic Pasteur Institute** internal research grant and a Bilateral Research & Technology Collaboration Greece-France 2013 grant (no 1811) funded by the Greek General Secretariat for Research and Technology (2013-2014)